EXHIBIT D

10250709 PMID: 7527602
Disruption of mitochondrial calcium homeostasis following chronic doxorubicin administration.

Solem L E; Henry T R; Wallace K B
Department of Pharmacology, University of Minnesota, Duluth 55812.
Toxicology and applied pharmacology (UNITED STATES) Dec 1994 , 129 (2) p214-22 , ISSN: 0041-008X--Print Journal Code: 0416575
Publishing Model Print
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Doxorubicin (Adriamycin) is an anthracycline antibiotic with broad antineoplastic activity. However, the clinical success is limited by the incidence of cumulative cardiomyopathy. In vitro, doxorubicin elicits a cyclosporine A-sensitive release of calcium from cardiac mitochondria. It has been suggested that this leads to mitochondrial calcium cycling and depolarization of membrane potential, which may account for the inhibition of mitochondrial respiration and cytotoxicity observed with the drug. Implication of a similar mechanism in the manifestation of clinical doxorubicin toxicity requires evidence for a disruption of mitochondrial calcium homeostasis following chronic in vivo administration. Cardiac mitochondria isolated from doxorubicin-treated rats (2 mg/kg/week, s.c. x 13 weeks) had a lower RCR but no change in ADP/O compared to controls and exhibited an enhanced cyclosporine A-sensitive release of mitochondrial calcium. Associated with this was a calcium-induced depolarization of membrane potential, which was inhibited by either cyclosporine A or ruthenium red suggesting the induction of mitochondrial calcium cycling following chronic doxorubicin treatment. The persistence of these effects on mitochondrial calcium regulation 4-7 days after the last drug treatment is consistent with the cumulative cardiotoxicity associated with doxorubicin therapy. Cardiac mitochondria isolated from rats treated with iminodaunorubicin, a noncardiotoxic analog of doxorubicin, showed no differences from control suggesting that this disruption of mitochondrial calcium homeostasis in vivo may be an important determinant of the cardiomyopathy observed clinically with doxorubicin.

09613124 PMID: 8386192
Doxorubicin cardiomyopathy is associated with a decrease in calcium release channel of the sarcoplasmic reticulum in a chronic rabbit model.

Dodd D A; Atkinson J B; Olson R D; Buck S; Cusack B J; Fleischer S; Boucek R J
Division of Pediatric Cardiology, Vanderbilt University Medical Center, Nashville, Tennessee 37232.
Journal of clinical investigation (UNITED STATES) Apr 1993 , 91 (4) p1697-705 , ISSN: 0021-9738--Print Journal Code: 7802877
Contract/Grant No.: CA 48930; CA; NCI; F32 HL08006; HL; NHLBI; HL32711; HL; NHLBI
Publishing Model Print
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Doxorubicin is a highly effective cancer chemotherapeutic agent that produces a dose-dependent cardiomyopathy that limits its clinical usefulness. Clinical and animal studies of morphological changes during the early stages of doxorubicin-induced cardiomyopathy have suggested that the sarcoplasmic reticulum, the intracellular membrane system responsible for myoplasmic calcium regulation in adult mammalian heart, may be the early target of doxorubicin. To detect changes in the calcium pump protein or the calcium release channel (ryanodine receptor) of the sarcoplasmic reticulum during chronic doxorubicin treatment, rabbits were treated with intravenous doxorubicin (1 mg/kg) twice weekly for 12 to 18 doses. Pair-fed controls received intravenous normal saline. The severity of cardiomyopathy was scored by light and electron microscopy of left ventricular papillary muscles. Developed tension was measured in isolated atrial strips. In subcellular fractions from heart, [3H]ryanodine binding was decreased in doxorubicin-treated rabbits (0.33 +/- 0.03 pmol/mg) compared with control rabbits (0.66 +/- 0.02 pmol/mg; $P < 0.0001$). The magnitude of the decrease in [3H]ryanodine binding correlated with both the severity of the cardiomyopathy graded by pathology score (light and electron microscopy) and the decrease in developed tension in isolated atrial strips. Bmax for [3H]ryanodine binding and the amount of immunoreactive ryanodine receptor by Western blot analysis using sequence-specific antibody were both decreased, consistent with a decrease in the amount of calcium release channel of sarcoplasmic reticulum in doxorubicin-treated rabbits. In contrast, there was no decrease in the amount or the activity of the calcium pump protein of the sarcoplasmic reticulum in doxorubicin-treated rabbits. Doxorubicin treatment did not decrease [3H]ryanodine binding or the amount of immunoreactive calcium release channel of sarcoplasmic reticulum in skeletal muscle. Since the sarcoplasmic reticulum regulates muscle contraction by the cyclic uptake and release of a large internal calcium pool, altered function of the calcium release channel could lead to the abnormalities of contraction and relaxation observed in the doxorubicin cardiomyopathy.

08179988 PMID: 2778802
Doxorubicin-induced calcium release from cardiac sarcoplasmic reticulum vesicles.

Kim D H; Landry A B; Lee Y S; Katz A M
Department of Medicine, University of Connecticut Health Centre, Farmington 06032.
Journal of molecular and cellular cardiology (ENGLAND) May 1989 , 21 (5) p433-6 , ISSN: 0022-2828--Print Journal Code: 0262322
Contract/Grant No.: HL-33026; HL; NHLBI
Publishing Model Print
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Doxorubicin, an anthracycline glycoside antibiotic which has been widely used for treatment of several types of cancer (Goormaghtigh and Ruysschaer, 1984), displays a clinically important cardiac toxicity (Young et al., 1981) that can be dissociated from the antitumor activity. Although the main sites of toxicity have been postulated to be on the muscle membranes (Goormaghtigh and Ruysschaer, 1984; Harris and Doroshow, 1985), no information is available for a direct doxorubicin effect on the Ca2+ fluxes in cardiac sarcoplasmic reticulum (SR). Previous studies have shown that micromolar doxorubicin triggers Ca2+ release from skeletal SR vesicles (Zorzato et al., 1985). The objective of this study was to examine the effect of doxorubicin or caffeine on Ca2+ fluxes in cardiac SR in the presence of various Ca2+ release inhibitors. Addition of either doxorubicin (C1/2 = 5 microM), or caffeine (C1/2 = 0.8 mM) triggered Ca2+ release from canine cardiac SR loaded with 45Ca2+ in the presence of 2 mM ATP. The maximal amount of Ca2+ release triggered by doxorubicin (38% of the total loaded Ca2+) was significantly higher than that released by caffeine (25%). Plots of the amount of Ca2+ release triggered by 20 microM doxorubicin or 2 mM caffeine vs. free Ca2+ concentration were a bell-shaped, with maximal Ca2+ release at 0.2 microM Ca2+. Ca2+ release triggered by either 20 microM doxorubicin or 2 mM caffeine was inhibited by ruthenium red (0.1 to 2 microM), ryanodine (1 to 100 microM) or tetracaine (0.1 to 1 mM), whereas 2 mM caffeine did not further activate Ca2+ release triggered by 50 microM doxorubicin, suggesting that the drugs may share the same Ca2+ release channel.